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A Statistical Analysis of *Neanthes arenaceodentata*, Sister Chromatid Exchange Data

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ABSTRACT

This paper presents a statistical evaluation of the sister chromatid exchange (SCE) assay using Neanthes arenaceodentata. Specific issues discussed are distribution of baseline SCE frequencies, transformations of SCE data, statistical sensitivity of the assay, repeatability of test results and sources of variability of SCE data. Probabilities of false positives and false negatives are considered and recommendations are made for appropriate transformation of data, experimental design, and test conditions.

INTRODUCTION

The importance of genetic effects in marine pollution studies has been recognized only recently. The International Council for the Exploration of the Seas dedicated a section of their 1979 Workshop on biological effects of marine pollution to genetics (1). They registered concern and recommended increased research efforts in this area.

To determine whether mutagenic compounds pose real threats to the marine environment, information is needed about the presence, distribution and abundance of such toxicants in the marine environment, the degree of genetic damage suffered by marine organisms, and the ecological consequences of such damage. No single test can address all of these concerns. However, at present, no genetic tests are included in permit programs that manage waste disposal in our estuarine, coastal and oceanic environments. Short-term tests are needed to identify mutagens and to evaluate complex mixtures. Long-term tests are needed also to investigate possible effects of somatic and germinal mutations on populations.

We are developing an *in vivo* assay to address these needs using the cytogenetic response of sister chromatid exchange (SCE) in the marine worm, *Neanthes arenaceodentata*. The application of SCE to the worm has created a new tool that is both relevant and practical to study genetic problems in marine environments. The SCE response has been recommended for environmental application by EPA's GENE-TOX Program (2). The worm *N. arenaceodentata* is used routinely in marine toxicology. Previous results indicate that this worm can metabolize promutagens and suggest that it may be sensitive to a broad spectrum of genetic toxicants (3). With the *in vivo* SCE assay, complex wastes can be tested under conditions which simulate real world situations.

Our goal in this paper is to examine the statistical aspects of this assay method. Previously, Latt et al. (2) have discussed the necessity of insuring that statistical criteria be met by SCE data and whether or not data need be transformed prior to analysis. Parametric statistical procedures, such as Analysis of Variance, assume that the data are normally distributed and that the variances are equal for all treatments being compared. These fundamental assumptions have been examined for SCE data in two different test systems. Dixon and Clark (4) reported that for adult blue mussels, Mytilus edulis, SCE data follow a Poisson distribution for individual animals. However, SCE counts for single cells from randomly selected animals within each treatment indicated the presence of variability between animals. They corrected for these problems by summing the SCE/cell counts for each animal and then applied a square root transformation. This stabilized the variance for all treatments and permitted parametric statistical analysis of the data. Carrano and Moore (5) have provided an extensive evaluation of SCE data from human lymphocytes. They found that no single family of probability distributions could be used to describe all of their data sets. Consequently, they applied a nonparametric test for statistical comparisons.

In this paper, we discuss distribution of baseline SCE observations, transformations of SCE data, statistical sensitivity to detect differences, sources of variability of SCE data, and repeatability of test results.

MATERIALS AND METHODS

Neanthes arenaceodentata Moore (Nereis acuminata Ehlers, Nereis caudata Delle Chiaje) construct mucous tubes within sediment or on firm surfaces. Females deposit eggs within the mucous tubes, then die; males incubate the eggs, and larvae develop within the tubes. At 20°C an entire

life cycle (egg to egg) is completed in 9 to 10 wk. All of the N. arenaceodentata larvae used in this study were obtained from laboratory cultures that originated at California State University, Long Beach. Details of culture techniques have been published by Reish (6).

For these experiments, 12 to 14 day old larvae were exposed in the dark to 1 X 10^{-5} M (3 mg/L) 5-bromodeoxyuridine (BrdU) in 100 ml of filtered seawater (30 o/oo salinity) at 20°C for 48 h unless noted otherwise. Colchicine (0.05%) was added to the seawater for the last 4 h of the 48 h exposure. Approximately 50 larvae per treatment were homogenized via acetic acid digestion and then applied to hot slides. Slides were prepared and examined as described in Pesch et. al, (3).

Mitomycin C (MMC) and BrdU were used to elicit dose response curves. The MMC was mixed to desired concentrations with filtered seawater, then was added concurrently with the BrdU. To obtain the BrdU dose response curve, the normal 48 h assay was run with higher concentrations of BrdU. SCE observations were made on 25 second-division cells for each treatment except for the highest concentration of mitomycin C (MMC) were the three separate experiments had sample sizes of 6, 10 and 5 respectively. The influence of cell cycle time on SCE response was checked by exposing larvae for 24, 48, and 72 h under control conditions. Also, clutch to clutch variation (a clutch is all larvae from a single pair of worms) was examined by assaying equal numbers of larvae from several clutches as well as from a pooled sample of these clutches tested under control conditions.

RESULTS

Initially, we pooled all baseline (control) observation (BrdU = 3 mg/L) data and looked at the variable X = SCE's/chromosome. There were

447 metaphase cell observations in this data set and their frequency distribution is given in Figure 1A. This distribution is skewed to the right and clearly does not follow a normal distribution. The overall mean and variance are 0.18 and 0.03, respectively. A sampling study was conducted to examine the influence of sample size on the distribution of means. Figure 2A,B,C gives the frequency distribution of 500 means based upon sample sizes of 5, 25, and 50 respectively. These distributions become less skewed and approach a normal distribution as n increases, as expected by the Central Limit Theorem of Statistics. However, only for samples of sizes $n \geq 45$ ($\alpha = .05$) was the distribution of the means found to follow a normal distribution. Based upon these results, a transformation of the SCE/chromosome data seems appropriate prior to its statistical analysis.

To determine the most appropriate transformation, we plotted the mean SCE's/chromosome for all our experimental treatments (63, ~25 observations/treatment) against their standard deviation. Figure 3A shows these results. Except for the three points, each with a mean >3, there is a very good relationship between the standard deviation and the mean, further evidence that a transformation of the data is appropriate. Figures 3B and 3C are plots of means and standard deviations for square root transformed and log (X + .1) tranformed data respectively. The constant (.1) was added because the log (0) is undefined. The square root transformation still shows a linear trend, whereas the log transformation reduces this trend.

Figure 1B shows the frequency distribution of log (X + .1) for the 447 baseline observations. A sampling study of the mean of the transformed data (500 means, n = 5) was conducted and shows that the assumption of normal distribution cannot be rejected for n as small as 5 (Fig. 2D).

Since the log (X + .1) distributes normally and displays equal variances amongst treatments, this seems to be the appropriate transformation.

To determine the sensitivity of our assay, we used the following formula (7):

$$\delta = (C\ 2\ \sigma^2/n)^{1/2}$$

where δ is the magnitude of difference that can be detected between two independent samples; C is a constant dependent upon choice of probability of Type I and Type II errors; n is the sample size per treatment and $2\ \sigma^2$ is the estimate of variance of the difference between 2 treatments. Table 1 gives the approximate magnitude of δ in log units; δ^* is the approximate difference in untransformed SCE's/chromosome. The probability of committing a type I error was fixed at .05 and we chose to use a one-sided test to detect only an increase in SCE's/chromosome, σ^2 = .0664. The probability of committing a type II error is 1 - Power of test. The power was fixed at 0.80, 0.90 and 0.95.

To illustrate the meaning of this calculation look at the last row (n=25) of table 1 for power = .80. The overall mean SCE's/chromosome, using log (X + .1) transformation for the baseline was -.63, approximately .23 SCE's/chromosome (untransformed). For transformed data we have an 80% chance of statistically detecting an increase of .18 in log units, that is a mean value of -0.45 or larger in log units different from the baseline. This corresponds to approximately a 0.12 increase in SCE's/chromosome over baseline for the raw data.

The data from Figure 4 allow us to compare the sensitivity of this system at two concentrations of BrdU, 15 mg/L (on the plateau of the curve) and 3 mg/L (on the slope of the curve). Table 2 gives an estimate of variance based on pooled data from two experiments for both concentrations of BrdU. We calculated (in log units) the sample size (n) necessary to

detect an increase of 0.20 SCE's/chromsome (Power = .9, α= .025), one-sided test (7). This increase of 0.20 SCE's/chromosome is approximately equivalent to a doubling of the mean SCE's/chromosome for the 3 mg/L BrdU concentration. The power to detect an increase of 0.20 SCE's/chromosome for the 15 mg/L BrdU concentration using 30 observations is 0.62 versus 0.90 for the 3 mg/L BrdU concentration.

Sources of variation in baseline SCE frequencies considered were time to harvest and clutch differences. The influence of time to harvest was tested by exposing organisms to control conditions for 24, 48 and 72 hours. There was no significant difference among the means for these three exposure durations. The means were: -.69, -.68, and -.67 for 24, 48 and 72 h, respectively. Clutch to clutch differences were tested by keeping worms from the same clutch separate and comparing their response with that of a pooled sample from several clutches. Besides the mean response, it is also important to look at the variance to see if the variance from an individual clutch is more consistent that the variance from a pool of several clutches. Table 3 gives the means and variances for two experiments. Experiment 1 consisted of a pool of 3 clutches, and individuals from each of those clutches. Experiment 2 was a pool of two clutches and individuals from each. There were no significant differences among the mean responses with either experiment. The variances are very consistent and do not indicate that within clutch variance is any less than that from a pool of worms from several clutches.

Reproducibility of dose response curves was tested by repeating dose response experiments, at different times. BrdU was tested twice, and MMC was tested three times. Figures 4A and 4B show the mean responses for repeated experiments to BrdU and MMC, respectively. An analysis of covariance was run on the results for each compound to test the hypothesis

of equality of slopes and intercepts of the dose responses for each repeated experiment. For neither compound was the hypothesis of equal slopes, or equal intercepts rejected ($\alpha = .05$). Analysis was run using approximately 25 observations per dose and log (X + .1) transformation.

DISCUSSION

A transformation of the N. arenaceodentata SCE data is required because of its skewed distribution and because there is a definite relation between the mean of a treatment and its standard deviation. Although Latt et. al. (2) mention several possible transformations, we conclude that the log transformation is appropriate for counts of SCE's/chromosome with N. arenaceodentata. It removes the relationship between the mean and the standard deviation and means based upon sample sizes as small as 5 are normally distributed.

A primary concern is the statistical sensitivity of the N. arenaceodentata SCE assay to detect an increased SCE response over baseline. The sensitivity of the method is evaluated by calculating the associated statistical power of the test to detect an increase in SCE frequency. Our assay system is used to test the hypothesis that the mean response of the controls is greater than or equal to the mean response of a dose treatment. Under this hypothesis, type I statistical error concludes there is a dose mean effect greater than the control mean when in reality this is not true, i.e., a false positive. Type II statistical error concludes that the dose mean effect is no greater than the control mean, when in reality the dose mean effect is greater, i.e. a false negative. The power of a statistical test is defined to be 1 - the

probability of making a type II error. The probability of type I error is the preselected level of significance of a statistical test, typically P = .05. The probability of type II error (and power) is a function of the differences to be detected, the variance of the mean response, and sample size. A two-fold increase over baseline SCE frequencies is recommended to indicate a positive response (2). To increase sample sizes is labor intensive with the N. arenaceodentata SCE assay. Therefore, the simplest approach to increase the power of the test is to decrease the variance of the mean baseline response. Carrano and Moore (5) examined this problem thoroughly and concluded that in the human lymphocyte system the "major source of inherent variation in baseline SCE frequencies can be attributed to the amount of BrdU present in the culture medium relative to the number of lymphocytes initially added." They dealt with this problem by selecting, as baseline, a BrdU concentration that was in the middle of the plateau region of the dose response curve. The plateau of the curve is by definition a region of minimum slope, so variations in BrdU concentration or cell numbers have minimal impact on baseline SCE frequencies.

BrdU elicits a dose response in N. arenaceodentata as in other test systems (8,9). However, with N. arenaceodentata the variance associated with the mean response increases dramatically in the plateau region of the curve. Therefore, a baseline BrdU concentration selected from the plateau region would reduce greatly the power of a statistical test to detect increases above baseline. An examination of our data indicates that the sensitivity of the assay is enhanced greatly using a baseline concentration of 3 mg/L BrdU vs. 15 mg/L. The accepted protocol (2) of using 25 counts per replicate is sufficient to detect a doubling of the mean response using 3 mg/L BrdU as a baseline concentration. The power to detect this doubling

is 0.9. A baseline concentration of 3 mg/L BrdU allows for a more sensitive statistical test because of a lower variance of the mean.

Other sources of variation in baseline SCE frequences considered were time to harvest and clutch to clutch differences. Neither of these proved to be a significant source of variation. In several studies, time to harvest or culture time was found not influence baseline SCE frequency (10,11,12). However, Snope and Rary (13) demonstrated a significant influence of culture time on SCE frequencies in cultured human lymphocytes. They hypothesize that different lymphocyte populations have different SCE frequencies. This raises the concern that alterations in cell replication kinetics induced by a toxicant may create an apparent change in SCE frequences. In test systems such as N. arenaceodentata (whole animal) which involve a complexity of cell types, the affect of toxicants on cell replication kinetics and SCE response needs careful consideration.

Ideally, one should design an experiment that has a fixed probability of making a type I error and the same probability of making a type II error, because guarding against false negatives is at least as important as guarding against false positives. We have achieved this level of sensitivity in the N. arenaceodentata SCE assay by identifying exposure sources of variation, by applying the log transformation to SCE's/chromosome data, and by using a BrdU concentration of 3 mg/L as a baseline level.

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Table 1. Increases in SCE's/chromosome detectable at powers of 0.80, 0.90, and 0.95 and sample sizes of 5, 10, 15, 20, and 25, $\alpha = 0.05$. Based on 447 control cell observations, δ = log 10 units, $*\delta$ = approximate difference in untransformed SCE's/chromosome, overall mean is 0.23 SCE's/chromosome (untransformed).

	POWER					
	.80		.90		.95	
n	δ	$*\delta$	δ	$*\delta$	δ	$*\delta$
5	.41	.37	.48	.48	.54	.58
10	.28	.22	.33	.27	.38	.33
15	.24	.18	.28	.22	.31	.24
20	.21	.15	.24	.17	.27	.21
25	.18	.12	.22	.16	.24	.18

Table 2. Sample size (n) necessary to detect an increase of 0.20 SCE's/chromosome (log units), α=0.025, one-sided test. Variance based on pooled data from two experiments for two concentrations of BrdU. Power at 3mg/L = 0.90, at 15 mg/L = 0.62.

BrdU (mg/L)	3	15
$\bar{x}$	-.568	-.076
s^2	.0563	.1094
n	30	58

Table 3. The means and variances for two experiments examining clutch to clutch differences in SCE's/chromosome response under control conditions.

		Mean	Variance
Experiment 1	Pool	-.58	.0731
	clutch 1	-.49	.0453
	clutch 2	-.44	.0764
	clutch 3	-.60	.0802
2	Pool	-.71	.0720
	clutch 1	-.72	.0729
	clutch 2	-.69	.0572

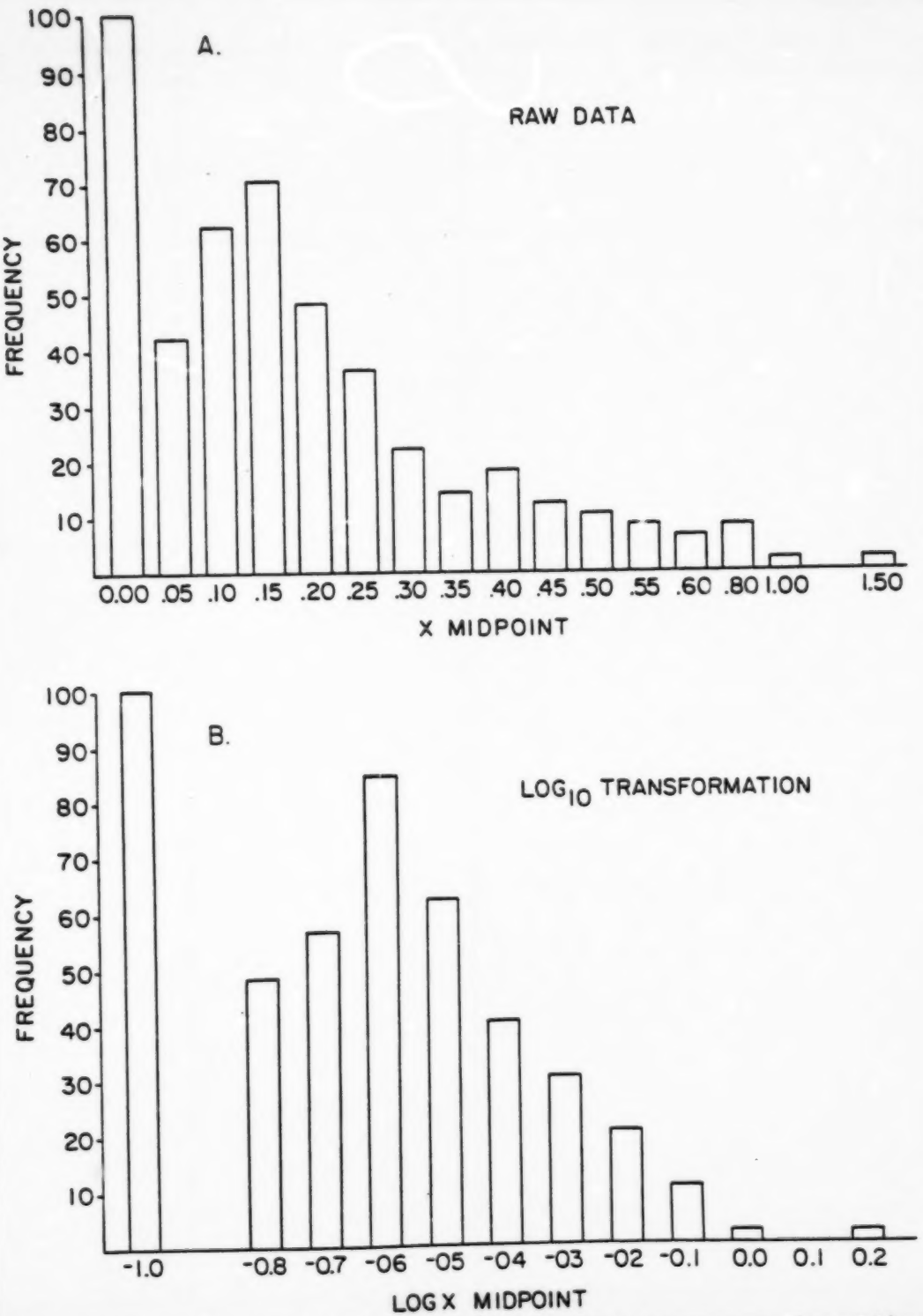

Figure 1. Frequency distributions of baseline SCE's/chromosome observations in 447 metaphase cells on Neanthes arenaceodentata; A raw data, B log transformed data.

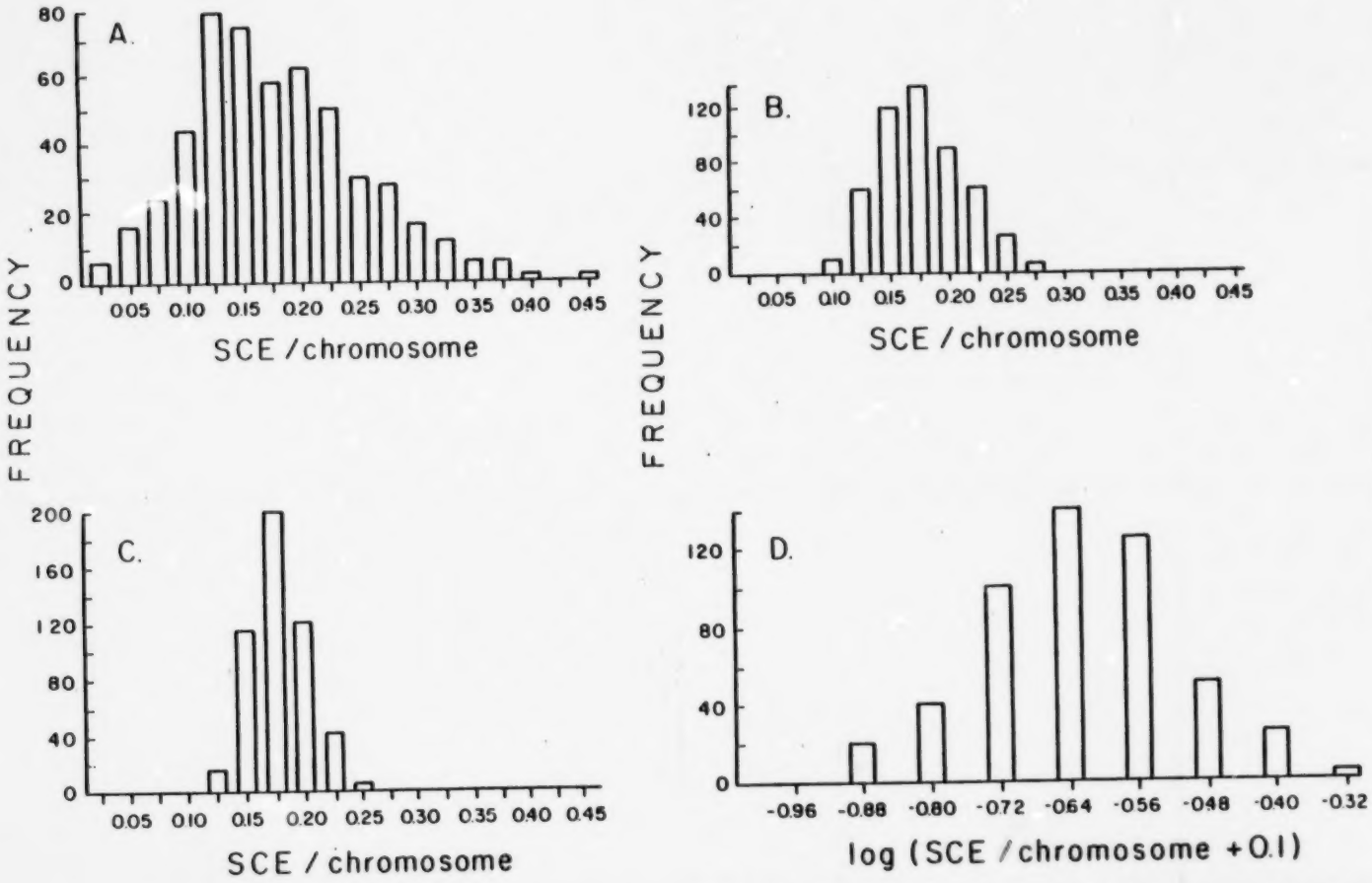

Figure 2. Frequency distributions of 500 sample means of SCE's/chromosome; A raw data, means based on sample size of 5, B raw data, means based on sample size of 25, C raw data, means based on sample size of 50, D log transformed data, means based on sample size of 5.

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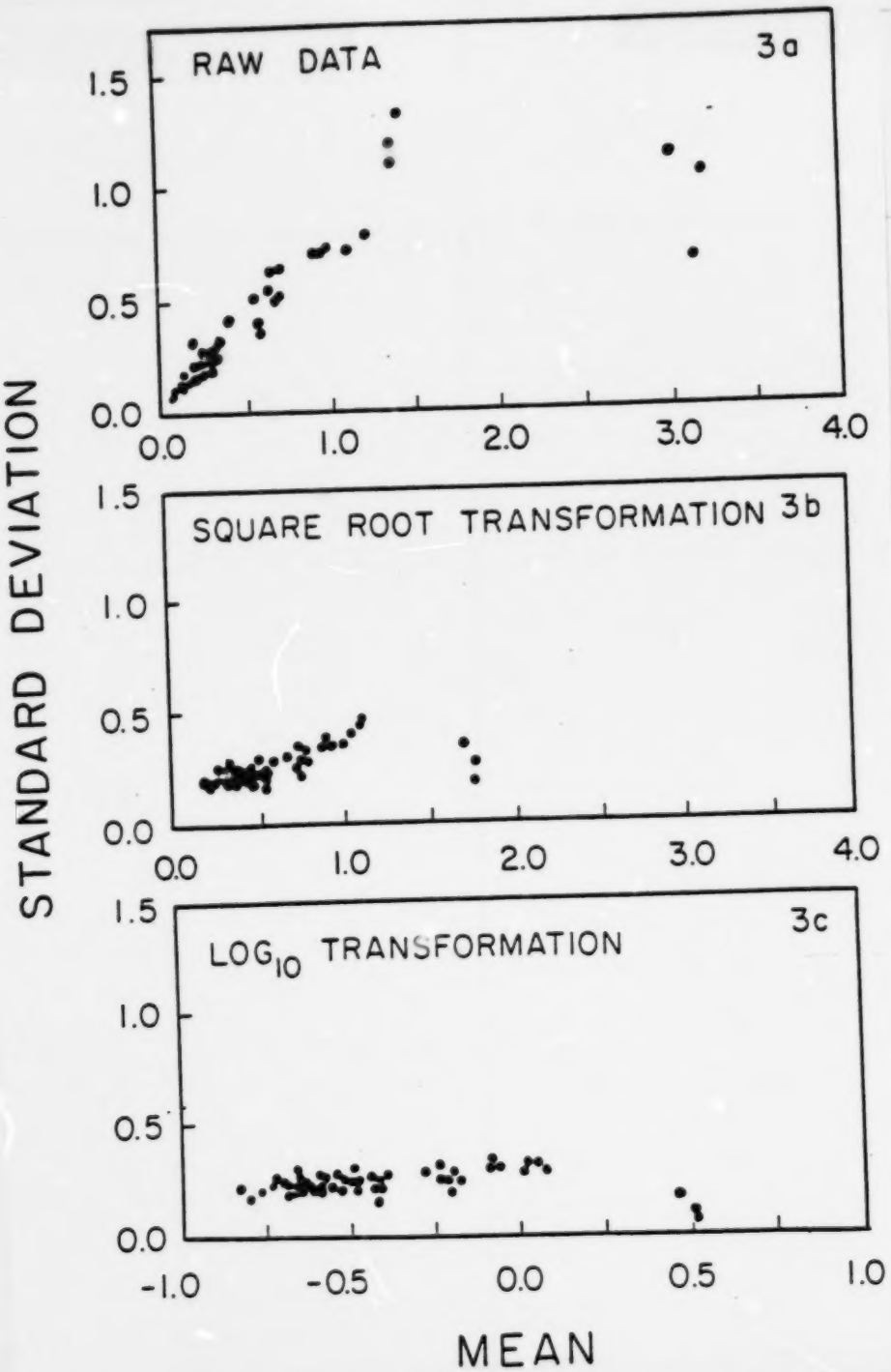

Figure 3. Plot of sample standard deviation versus sample mean SCE's/ chromosome for Neanthes arenaceodentata for 63 experimental treatments.

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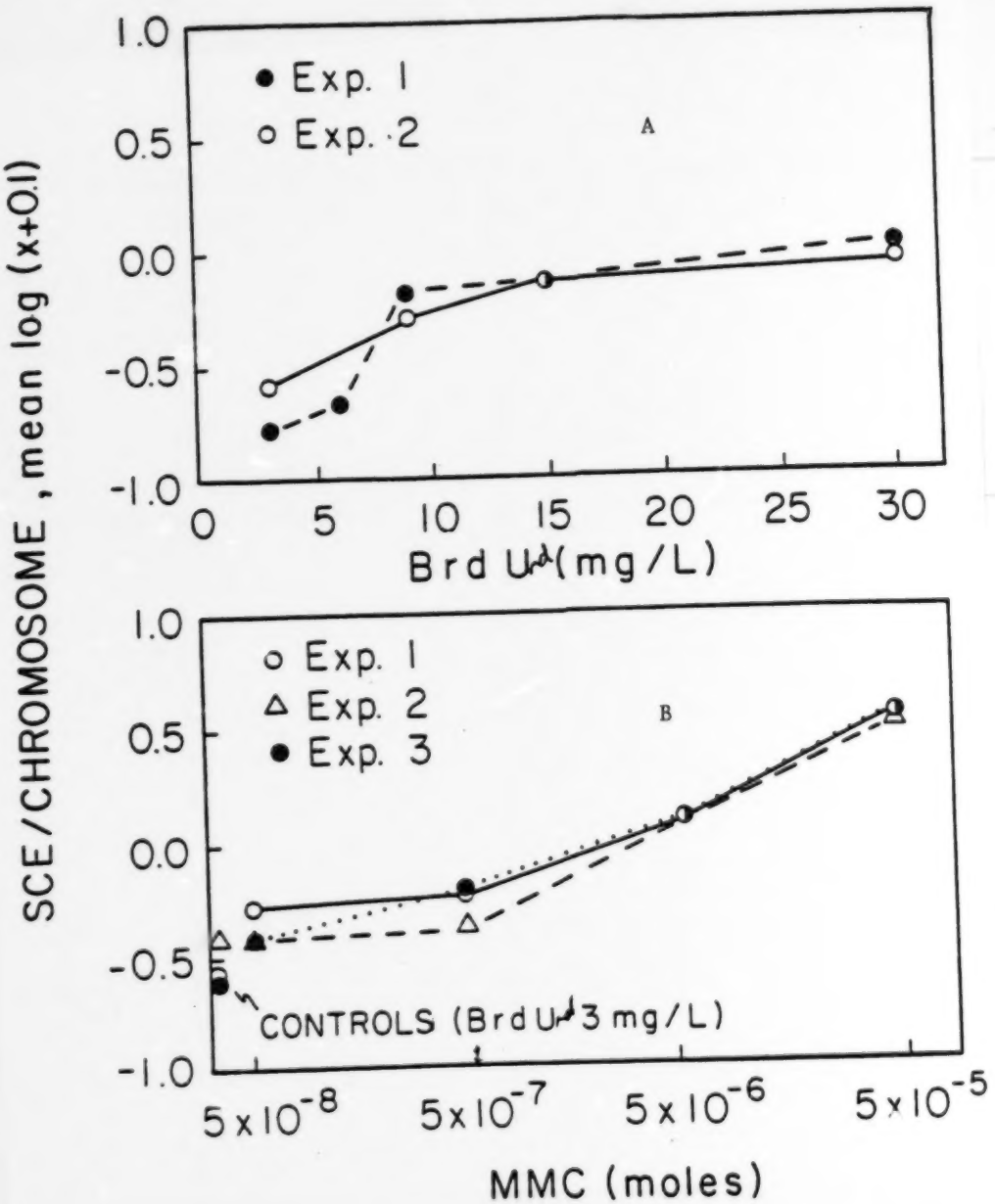

Figure 4. Replicate dose response experiments measuring SCE's/chromosome in Neanthes arenaceodentata.